2.4. Intensity Measurement and Ratio Calculation

Upon obtaining the correct target site, the measurements of fluorescent intensities from both fluorescent channels for a target region are obtained in the following manner:

For each detected target with total of n pixels, $p_1, p_2, \ldots, p_n$,

1. Sort $p_1, \ldots, p_n$ against their first fluorescent intensity, and trim top 5% and bottom 5% to obtain $p'_1, \ldots, p'_{n_1}$, where $n_1 = 0.9n$.
2. Sort $p_1, \ldots, p_n$ against their second fluorescent intensity, and trim top 5% and bottom 5% to obtain $p''_1, \ldots, p''_{n_1}$,
3. Select pixel set, $\{p^*_1, \ldots, p^*_{n_2}\}$ such that $p^*_j \in \{ p'_1, \ldots, p'_{n_1}\} \cap \{ p''_1, \ldots, p''_{n_1}\}$. This step guarantee that a selected pixel is not in the 5% trimmed set in both steps 1 and 2, and it may effectively trim up to 10% of the pixels from top and up to 10% from the bottom, or $0.9n \leq n_2 \leq 0.8n$, depending on the intersection.
4. Calculate mean intensities, standard deviations and other measurement statistics from both fluorescent channels and corresponding expression ratios based on the pixel collection $\{p^*_1, \ldots, p^*_{n_2}\}$. Also calculate the total intensities from both channels based on the original pixel set $\{p_1, \ldots, p_n\}$.

The trimmed mean is used for estimating the average intensity to lessen the interference of dust particles or other spike noises, as well as possible mis-registration between the two fluorescent channels. Noise spikes formed from various sources are usually small but with high fluorescent intensity. Trimming suppresses most of them, while maintaining the robustness of the averaging process and compatibility with the background level estimation. We take the background subtracted mean intensity, which possesses a normal distribution when large numbers are presented (central limit theorem), as the estimate of signal intensity. This normality assumption for the mean measurement forms the basis for using simulations to study properties of expression ratios derived from the two mean intensities.